SYNOPSIS OF RESEARCH PROJECT

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4.	**Name of the Degree Submitted for**	**:**	Ph.D. (Agri.)
5.	**Major Subject**	**:**	Molecular Biology and Biotechnology
6.	**Minor Subject**	**:**	Biochemistry
7.	**Name of Major Guide**	**:**	Dr. H. P. GAJERA Professor & Head, Dept. of Biotechnology, COA, JAU
8.	**Title of Research Project**	**:**	**MOLECULAR DISSECTION OF GRAIN IRON AND ZINC BIOFORTIFICATION IN BREAD WHEAT USING PHENOTYPIC, GENOTYPIC AND GENE EXPRESSION APPROACHES**

Introduction

Wheat (*Triticum aestivum* L.), a cool-season cereal that is self-pollinated, hexaploid (2n = 6x = 42), and the second most important food grain after rice. One of the main basic foods in India, wheat is essential to the country's food and nutritional security. Grain iron content (GFeC) and grain zinc content (GZnC) have been found to vary genetically in wheat landraces, and wheat grains are rich in carbohydrates, proteins, vitamins, and minerals like iron (Fe) and zinc (Zn). from 24.93 to 66.51 and 18.68–38.66 ppm respectively (Heidari *et al*., 2016) However, Due to anti-nutritional elements like phytic acid, which restrict their absorption in the human body, these micronutrients frequently have low concentrations and bioavailability. Wheat is primarily grown under diverse agro-climatic conditions, including irrigated and rainfed environments. However, its productivity is often affected by abiotic stresses such as drought, heat stress, and changing climatic conditions, which are becoming more frequent and severe due to climate change is one of the most important crops

worldwide, and, as a resilient cereal, it grows in various climatic zones. Due to changing climatic conditions and naturally occurring environmental fluctuations, the priority problem in the cultivation of wheat is to improve the quality of the crop. Biotic and abiotic stressors are known factors leading to the deterioration of wheat grain quality and to crop yield reduction. India, one of the greatest success stories of Green Revolution, is the second largest producer of wheat in the world with production hovering around 70– 75 million tons in the past few years (Nagarajan 2005). It accounts for approximately 12% of world's wheat production and is the second largest wheat consumer after China (FAO 2004)**.**

The current state of knowledge on wheat genetics shows significant progress in the analysis of gluten, starch, and lipid genes responsible for the synthesis of the main nutrients in the endosperm of common wheat grain (Filip *et al*.2023). In countries like India, wheat contributes significantly to daily caloric intake and plays a crucial role in ensuring food security. Providing approximately 20% of the world's protein and caloric intake (Sharma *et al*.2025) However, while wheat is a rich source of carbohydrates and energy,it is inherently deficient in essential micronutrients such as zinc (Zn) and iron (Fe). Over 3.0 billion people of the 7.75 billion globally suffer from malnutrition due to deficiencies in micronutrients, particularly iron, zinc, and vitamins (such as vitamin A, B complex, E, and folate, etc.). However, the dietary intake of these micronutrients could be sufficient to meet the nutritional demand if the bioavailability was not low due to the strong inhibition by phytic acid and phenolics.This nutritional limitation has led to widespread micronutrient malnutrition, commonly referred to as "hidden hunger."(varma *et al*.2021) Zinc and iron are essential for various physiological and biochemical processes in the human body, including immune function, oxygen transport, enzyme activity, and cognitive development. Their deficiency can lead to severe health problems such as anemia, stunted growth, weakened immunity, and increased susceptibility to infections. Recognizing the seriousness of this issue, international organizations such as the Food and Agriculture Organization and the World Health Organization have emphasized the need to enhance the nutritional quality of staple crops.

Biofortification has emerged as a promising and sustainable strategy to address micronutrient malnutrition by increasing the concentration of essential nutrients in crops through genetic improvement. Unlike conventional fortification methods, which require industrial processing, biofortification ensures that nutrients

are inherently present in the edible parts of crops. In wheat, biofortification of Zn and Fe is particularly important due to its widespread consumption. However, improving these micronutrients in wheat grains is challenging because their accumulation is controlled by multiple genes and influenced by environmental conditions such as soil nutrient availability and climate.(Singh *et al*. 2021)

Wheat is an important staple cereal providing major source of energy and protein across the world. However, there are several antinutritional factors present in wheat, such as phytate, protease inhibitor, tannins, lectins, alkaloids, oxalate, etc in wheat grains further reduces the bioavailability of Zn and Fe. Phytic acid binds with these minerals to form insoluble complexes, limiting their absorption in the human digestive system. Therefore, it is essential not only to increase the total concentration of Zn and Fe but also to assess their bioavailability.(Ram *et al*.2020) Although sufficient quantities of iron and zinc are available in cereal grains (Raboy 2001; Borg *et al*. 2009; Paul *et al*. 2013), the bioavailable Fe and Zn in staple food crop grains are as low as 5 and 25 %, respectively (Bouis and Welch 2010). Parameters such as phytase activity and molar ratios (phytate to Zn/Fe) are important indicators of micronutrient bioavailability.

Phenotypic evaluation of wheat genotypes plays a critical role in identifying lines with superior nourishing quality. Colored wheat is a valuable resource that is rich in anthocyanins and minerals and thus contributes additional nutritional value to a healthy human diet.(fan *et al*.2020) Thetransgenic crop sare being developd for reduced content of phytic acid (Paul *et al*. 2013;Frontelaetal. 2009) in grains. Breeding for nutritional quality is a viable, practicable, and cost-effective strategy to com plement existing interventionist strategies (Bouis and Welch2010; Clugstonand Smith2002).The problems that some varieties with high iron and zinc are tall, traditional and low-yielding (Bouis and Welch 2010; *Paul et al*. 2013). However, the objective is not only to increase the content of micronutrients in the edible part of the plant but also to increase their bioavailability which can be achieved if the anti-nutrient component (phytate in this case) can be degradedinsitu. Therefore, a long term solution using crop varieties with increased zinc and iron bioavailability is highly desirable Analysis of biochemical traits such as moisture, protein, gluten, starch, fat, Zn, Fe, and phytic acid provides valuable information about the nutritional profile of different genotypes. (kure *et al*.2020) Screening a diverse set of wheat genotypes, including biofortified lines, allows the identification

of promising candidates for further improvement. However, phenotypic traits are often influenced by environmental factors, which can limit the efficiency of selection based solely on observable characteristics

To overcome these limitations, molecular marker-based approaches have become essential tools in modern plant breeding. Among these, single nucleotide polymorphism (SNP) markers are widely used due to their abundance, stability, and high resolution. SNPs represent single base changes in the DNA sequence and are distributed throughout the genome. These markers are highly suitable for identifying genetic variation associated with complex traits such as Zn and Fe accumulation. SNP-based techniques, have facilitated the identification of genomic regions controlling micronutrient traits in wheat.(Zhou *et al*.2020). Kompetitive Allele-Specific PCR (KASP) is a fluorescence-based genotyping technology developed from SNP markers. It enables precise and high-throughput detection of allelic variation among genotypes. KASP markers are highly efficient, cost-effective, and suitable for large-scale screening of breeding populations. In wheat bio fortification programs, SNPs associated with Zn and Fe traits are often converted into KASP markers to facilitate marker-assisted selection (MAS). This approach allows breeders to rapidly identify and select genotypes carrying favorable alleles, thereby accelerating the development of improved varieties.(Yan *et al*. 2025)

While molecular markers provide information about genetic variation, understanding the functional role of genes requires gene expression analysis.(kumar *et al.* 2023) Gene-specific expression studies help in identifying genes that are actively involved in micronutrient uptake, transport, and storage within the plant. Techniques such as quantitative real-time PCR (qRT-PCR) are widely used to measure gene expression levels with high accuracy.(Chauhan *et al*. 2011) By comparing gene expression patterns between genotypes with high and low Zn and Fe content, it is possible to identify key genes responsible for enhanced micronutrient accumulation.(Lekshmy *et al*.2015)

In wheat, several candidate genes have been reported to play important roles in Zn and Fe homeostasis. These include genes encoding metal transporters such as ZIP (Zinc-regulated transporter/Iron-regulated transporter-like protein), NRAMP (Natural Resistance-Associated Macrophage Protein), and YSL (Yellow Stripe-Like) transporters. These proteins are involved in the uptake of micronutrients from the soil, their translocation within the plant, and their accumulation in the grains. Studying the

expression of these genes provides valuable insights into the molecular mechanisms underlying biofortification.

The integration of phenotypic evaluation, molecular marker analysis, and gene expression studies offers a comprehensive approach to improving micronutrient content in wheat. Phenotypic analysis helps in identifying superior genotypes, while SNP and KASP markers enable precise genetic characterization. Gene expression studies further validate the functional significance of candidate genes. This integrated strategy enhances the efficiency and accuracy of breeding programs aimed at developing biofortified wheat varieties.

In the present study, a set of wheat genotypes, including biofortified and normal lines, is evaluated for nutritional quality and micronutrient content. Biochemical analysis of grains is conducted to assess parameters such as moisture, protein, gluten, starch, fat, Zn, Fe, and phytic acid, along with phytase activity and bioavailability of micronutrients. Genotypic evaluation is performed using SNP/KASP markers at the seedling stage to identify genetic variation associated with Zn and Fe traits. Furthermore, gene-specific expression analysis using qRT-PCR is carried out on selected genotypes to understand the molecular basis of micronutrient accumulation. Thus, the present research integrates phenotypic, genotypic, and molecular approaches to address the challenge of Zn and Fe deficiency in wheat. The findings of this study are expected to contribute to the development of nutritionally enriched wheat varieties, thereby supporting efforts to combat hidden hunger and improve human health in a sustainable manner.

Practical Utility of the Research Project

This study focuses on the use of SNP/KASP markers and gene expression analysis for improving zinc (Zn) and iron (Fe) content in bread wheat. It has direct applications in crop breeding and nutritional improvement. KASP markers provide a fast, accurate, and low-cost method for identifying wheat genotypes with higher Zn and Fe levels. This helps breeders select superior plants at an early stage, reducing the need for repeated field and laboratory testing. As a result, the development of biofortified wheat varieties becomes quicker and more efficient. Gene-specific expression analysis helps in understanding how key genes control the uptake, transport, and accumulation of Zn and Fe in grains. This knowledge allows researchers to target important genes for improving micronutrient content through breeding or biotechnological approaches. The outcomes of this research can lead to

the development of nutrient-rich wheat varieties that help reduce micronutrient deficiency (hidden hunger), especially in populations dependent on wheat as a staple food. It also supports sustainable agriculture by combining high yield with improved nutritional quality. Overall, this approach saves time, reduces costs, and contributes to better human health and food security.

Objectives

1. To evaluate wheat genotypes for grain nutritional quality with emphasis on iron (Fe) and zinc (Zn) content (phenotypic evaluation).
2. To estimate anti-nutritional factors and assess the bioavailability of Fe and Zn in wheat grains.
3. To identify and validate SNP/ KASP markers associated with grain Fe and Zn content for efficient marker-assisted selection (genotypic evaluation).
4. To analyze the expression of candidate genes involved in Fe and Zn accumulation in wheat grains.
5. To elucidate the molecular mechanisms underlying grain Fe and Zn accumulation for the development of biofortified wheat varieties.

12. Review of Literature

12.1 grain nutritional quality and anti-nutritional factor in wheat

Velu *et al*. (2019) reported that improving grain iron (Fe) and zinc (Zn) concentration through biofortification is an effective strategy to reduce micronutrient malnutrition. They highlighted that modern wheat varieties (*Triticum aestivum*) have limited genetic variability for these nutrients, while higher levels are found in wild relatives such as *Triticum monococcum*, *Triticum dicoccoides*, and *Aegilops tauschii*. These genetic resources are used in breeding programs to develop high-yielding biofortified wheat with enhanced Zn and Fe content.

Gupta *et al*. (2020) studied that Biofortification and bioavailability of Zn, Fe and Se in wheat: present status and future prospects .Biofortification of wheat with micronutrients like zinc, iron, and selenium is an important research priority. Modern breeding for high yield has reduced grain nutritional quality, making improvement necessary. Recent studies focus on enhancing both nutrient content and bioavailability by evaluating genetic diversity and factors like phytic acid. Techniques such as QTL mapping, GWAS, and marker-assisted selection have helped identify important genes and markers. Research on nutrient uptake and storage, along with transgenic approaches, has supported this goal. Although some biofortified wheat varieties have

been developed, their large-scale adoption is still limited.

Kaur *et al*. (2020) evaluated nutritional and quality traits in biofortified bread wheat (*Triticum aestivum*) genotypes. Fifty genotypes were screened for grain iron (Fe) and zinc (Zn) content, and nineteen promising genotypes with higher micronutrient levels were selected for further biochemical and quality analysis. The study highlighted biofortification as an effective approach to improve the nutritional value of wheat.

Sharma *et al*. (2021) reported that iron (Fe) and zinc (Zn) deficiency is a major global nutritional problem, particularly in regions dependent on cereal-based diets. Wheat (*Triticum aestivum*) generally has low bioavailable Fe and Zn; therefore, biofortification through genetic improvement is an important strategy. The study highlighted the use of wheat wild relatives and landraces as valuable sources of genetic variability to enhance Fe and Zn content in wheat breeding programs.

Rabieyan *et al*. (2023) screened a large set of bread wheat genotypes under different environmental conditions to identify lines with better yield and nutritional traits. They observed considerable genetic variation for iron (Fe) and zinc (Zn) content, and selected promising genotypes combining good yield with improved nutrient levels for use in biofortification breeding.

Hakeem *et al*. (2025) investigated genetic diversity in wheat to improve grain nutrition and yield through biofortification. A large germplasm set including *Triticum aestivum*, *Triticum durum*, and × Triticosecale was screened to identify genotypes with higher iron (Fe) and zinc (Zn) content. The study emphasized the importance of utilizing diverse genetic resources to develop wheat varieties with improved micronutrient levels and better bioavailability.

Saha *et al.* (2017) reported that proper zinc (Zn) fertilization strategies can improve Zn content and its bioavailability in wheat (*Triticum aestivum*). The study showed that combining soil and foliar Zn application at suitable growth stages enhanced grain Zn levels and nutritional quality, while minimizing losses during processing.

Bilgarmi *et al*. (2018) reported that iron (Fe) and zinc (Zn) deficiency is a major nutritional concern worldwide. The study on wheat (*Triticum aestivum*) showed that micronutrient content is influenced by genotype and growing season, while phytic acid can reduce the bioavailability of Fe and Zn. The authors suggested that combining breeding approaches with molecular tools such as Single Nucleotide

Polymorphism and Kompetitive Allele Specific PCR markers can help develop wheat varieties with improved micronutrient content.

Garcia-oliveira *et al*.(2018)Studied that Genetic Basis and Breeding Perspectives of Grain Iron and Zinc Enrichment in Cereals. Micronutrient deficiency, or "hidden hunger," is a major global issue, especially in regions dependent on cereal-based diets. Iron (Fe) and zinc (Zn) are essential nutrients, but their levels and bioavailability in cereals are often low due to compounds like phytic acid. Advances in plant science have improved understanding of Fe and Zn uptake and movement in crops, though there is also a risk of toxic metal accumulation. Therefore, breeding nutrient-rich and safe cereal varieties is an effective strategy to improve human nutrition and reduce malnutrition.

Singh *et al*. (2018) reported that plant endophytes can improve growth, yield, and micronutrient accumulation in wheat (*Triticum aestivum*). The study found that certain bacterial strains such as Bacillus subtilis and Arthrobacter enhanced iron (Fe) and zinc (Zn) uptake, increased grain yield, and reduced phytic acid content, indicating their potential as bio-inoculants for improving wheat nutritional quality.

Hao *et al*. (2021) reported that foliar application of micronutrients can enhance iron (Fe) and zinc (Zn) content in wheat (*Triticum aestivum*). The study found that zinc foliar spray significantly increased Zn concentration and its bioavailability in grain, especially in wheat landraces, whereas foliar iron application showed limited improvement in Fe content.

Kumar *et al*. (2022) reported that pearl millet (*Pennisetum glaucum*) is a rich source of iron (Fe) and zinc (Zn), but the presence of anti-nutritional compounds such as phytates, fiber, and tannins reduces their bioavailability. The study suggested that biofortification and processing methods like soaking, fermentation, and heat treatment can improve micronutrient availability.

Saquee *et al*.(2023)Studied that The Efficacy of Micronutrient Fertilizers on the Yield Formulation and Quality of Wheat Grains. Micronutrient fertilizers are essential for improving wheat yield and grain quality, especially under changing climate conditions. Deficiencies of nutrients like zinc and iron lead to hidden hunger and poor health. Proper application, particularly through foliar methods, enhances plant growth, stress tolerance, and nutritional value. However, optimizing the dose and timing of micronutrient use is necessary for sustainable production.

12.2 SNP/ KASP markers for biofortification of Fe and Zn content in wheat

Shaukant *et al*. (2021) reported that evaluation of historical wheat cultivars (1911–2016) showed variation in grain iron (Fe) and zinc (Zn) content in wheat (*Triticum aestivum*). The study indicated that modern cultivars have higher yield but comparatively lower micronutrient levels, highlighting the need for breeding strategies that improve both yield and nutritional quality**.**

Basnet *et al*. (2022) reported that several quantitative trait loci (QTLs) and candidate genes are associated with iron (Fe) and zinc (Zn) accumulation in maize The study identified important gene families involved in micronutrient uptake and transport and suggested that marker-assisted breeding can be used to develop maize varieties with improved nutritional quality.

Tong *et al.(*2022) studied that High Resolution Genome Wide Association Studies Reveal Rich Genetic Architectures of Grain Zinc and Iron in Common Wheat. Biofortification is an effective approach to reduce micronutrient deficiency by increasing zinc and iron content in wheat grains. Genome-wide association studies identified several stable loci and new QTLs controlling these traits. Candidate genes involved in nutrient uptake, transport, storage, and regulation were also discovered. Additionally, KASP markers were developed for major QTLs, aiding in precise breeding. These findings enhance understanding of the genetic control of grain zinc and iron and support the development of nutrient-rich wheat varieties.

Wani *et al*. (2022) reported that biofortification of wheat (*Triticum aestivum*) with iron (Fe) and zinc (Zn) is an effective strategy to reduce micronutrient deficiency. The study highlighted that genomic approaches and gene families such as NAS gene family, ZIP gene family, VLT gene family, ZIFL gene family, and YSL gene family play an important role in improving micronutrient accumulation while maintaining grain yield.

Sun *et al*. (2023) reported the identification of stable QTLs associated with grain iron (Fe) and zinc (Zn) content in wheat (*Triticum aestivum*). The study detected several QTLs and candidate genes influencing micronutrient accumulation and grain weight, and validated KASP markers for their use in marker-assisted breeding to develop nutrient-rich wheat varieties.

Nasim *et al*. (2025) reported that micronutrient biofortification in wheat (*Triticum aestivum*) is an effective strategy to address hidden hunger caused by iron (Fe) and zinc (Zn) deficiencies. The study highlighted the role of QTL mapping, candidate genes, and molecular approaches such as marker-assisted selection and

advanced genomic tools in improving micronutrient content in wheat.

12.3. Gene expression analysis associated with grain Fe and Zn accumulation in wheat

Borg *et al*. (2012) reported that ferritin genes play an important role in iron accumulation in wheat (*Triticum aestivum*). The study identified two ferritin genes, TaFer1 and TaFer2, which regulate iron storage in the grain. Overexpression of the TaFer1 gene in the endosperm significantly increased grain iron content, indicating its potential for wheat biofortification.

Velu *et al*. (2014) reported that biofortification is an effective strategy to increase zinc (Zn) and iron (Fe) concentration in wheat (*Triticum aestivum*). The study highlighted that both genetic approaches using diverse germplasm and agronomic practices such as zinc fertilization can enhance grain micronutrient levels without affecting important agronomic traits.

Riaz *et al*. (2017) reported that iron (Fe) deficiency is a major global nutritional problem, particularly in populations relying on wheat-based diets. The study emphasized that biofortification of wheat (*Triticum aestivum*) through the use of genetic variation and breeding approaches can help improve grain iron content and reduce micronutrient malnutrition.

Kumar *et al*. (2019) reported that biofortification of crops with iron (Fe), zinc (Zn), and vitamin A is an effective approach to reduce micronutrient malnutrition. The study highlighted that while conventional breeding has limitations due to low genetic variability, transgenic approaches offer a precise and efficient method to develop nutrient-rich crop varieties.

Ludwig *et al*.(2019) reported that genetic biofortification is an effective strategy to enhance iron (Fe) content in major staple crops such as rice (Oryza sativa) and wheat (*Triticum aestivum*). The study highlighted that conventional and agronomic approaches have limitations, while genetic biofortification offers a sustainable solution to increase grain iron levels and reduce micronutrient malnutrition.

Ali *et al*. (2020) reported that genomic resources can accelerate biofortification in wheat (*Triticum aestivum*) to improve iron (Fe) and zinc (Zn) content in grains. The study highlighted the use of QTL mapping, genome-wide association studies, and advanced genomic tools such as gene editing and TILLING to identify genes associated with micronutrient accumulation for developing nutrient-

rich wheat varieties.

Akhtar *et al*. (2022) reported that iron (Fe) and zinc (Zn) biofortification improves grain quality and yield attributes in wheat (*Triticum aestivum*). The study showed that both soil and foliar application of micronutrients, particularly foliar spray at higher concentrations, significantly enhanced grain micronutrient content and yield performance.

12.4 Molecular mechanisms underlying grain Fe and Zn accumulation for the development of biofortified wheat varieties.

Xia *et al*. (2020) studied the source–sink relationship of zinc biofortification in wheat grains and reported that increasing Zn supply through soil or foliar fertilization and improving nutrient interactions can enhance Zn accumulation in wheat grain. The study also emphasized that efficient uptake, translocation, and remobilization of Zn from vegetative tissues to grain are important for achieving higher grain Zn concentration.

Gupta *et al*.(2021) Studied that Biofortification and bioavailability of Zn, Fe and Se in wheat: present status and future prospects. Biofortification of wheat with essential micronutrients such as iron (Fe), zinc (Zn), and selenium (Se) has become an important objective in wheat breeding programs. Modern breeding for higher yield has reduced micronutrient concentration in grains, which has affected their nutritional quality. Therefore, researchers are focusing on genetic evaluation, QTL/GWAS analysis, and marker-assisted selection to develop wheat cultivars with improved micronutrient content and bioavailability.

Kumaral *et al*. (2022) studied that genetic biofortification of wheat with zinc and reported that improving Zn uptake, transport, and grain loading is essential to increase zinc concentration in wheat grains. They also highlighted that molecular transporters such as ZIP and YSL regulate Zn movement and distribution in the grain, which plays an important role in developing Zn-biofortified wheat varieties.

Roy *et al*. (2022) Studied that Genomic approaches for improving grain zinc and iron content in wheat. Iron and zinc deficiencies affect billions of people worldwide, especially in developing regions such as South Asia and Sub-Saharan Africa. Biofortification of staple crops like *Triticum aestivum* through plant breeding is considered a sustainable approach to increase micronutrient intake in human diets. Studies have shown that improvement in mineral uptake, efficient source-to-sink translocation, and grain deposition can enhance Fe and Zn concentration in wheat

grains. Modern breeding approaches such as QTL mapping, marker-assisted selection, and genomic selection are being used to accelerate the development of biofortified wheat varieties with improved nutritional quality.

Jadon *et al*. (2023)Studied that Molecular Mapping of Biofortification Traits in Bread Wheat (*Triticum aestivum* L.) Using a High-Density SNP Based Linkage Map. A study using recombinant inbred lines derived from a cross between HD2932 and Synthetic 46 identified several QTLs associated with grain iron concentration, zinc concentration, protein content, and thousand kernel weight in *Triticum aestivum*. The research highlighted that these QTL regions contain candidate genes involved in Fe and Zn transport, grain development, and nutrient remobilization, which can be effectively utilized in marker-assisted selection to develop nutritionally improved wheat varieties.

13. Materials and Methods

I.	**Title of experiment**	:	**Molecular Dissection of Grain Iron and Zinc Biofortification in Bread Wheat using Phenotypic, Genotypic and Gene Expression Approaches**
II.	**Location**	:	Department of Biotechnology, College of Agriculture, J.A.U., Junagadh.
III.	**Crop**		Wheat (*Triticum aestivum L.*)
IV.	**Year and season of experiment**	:	2026-27
			Rabi
Experiment 1: Phenotypic evaluation of wheat genotypes for grain nutritional quality and antinutritional factor			
1	**Location**		Department of Biotechnology, College of Agriculture, J.A.U., Junagadh.
2	**Crop and genotypes**		Wheat (*Triticum aestivum L.*) –approximately 30 genotypes as per availability and suggestions by Research Scientist (Wheat)
3	**No. of replications**		3
4	**Statistical design**		CRD

5	**Sampling**	**Mature seeds of wheat procured from** Wheat Research Station, JAU, Junagadh
6	**Observations to be recorded**	
6a	**Morphological parameters**	
1.	**Thousand Grain Weight (g) (Test weight)**	Thousand grain weight will be determined by counting 1000 grains from each genotype and weighing them using an electronic precision balance. The result was recorded in grams. (Food and Agriculture Organization, 2011).
2.	**Grain Colour**	Grain colour of wheat **will be determined** by visual observation. (International Maize and Wheat Improvement Center, 1985).
3.	**Grain Volume**	Grain volume of wheat can be calculated by considering the grain as an ellipsoid using the following formula: V= 3/4 πabc (Breseghello *et al*.2007)
4.	**Grain Hardness**	Grain hardness **will be evaluated** based on kernel texture using the **Single Kernel Characterization System (SKCS)**. (American Association of Cereal Chemists, 2000).
6b	**Biochemical Parameters**	
1	**Moisture (%)**	Fresh weight and dry weight will be carried out for moisture percentage by oven dray method (AOAC. 2012)
2	**Protein content**	Protein content of control and selected transgenic lines will be determined according to the methods of AOAC. (2012)
3	**Gluten content**	Gluten content of control and selected transgenic lines will be determined according to the methods of AOAC. (2012)
4	**Starch content**	Starch content will be estimated following the method of Pearson. (1991)
5	**Fat content**	Fat content of control and selected transgenic lines will

		be determined according to the methods of AOAC. (2012)
6.	**Fiber content**	Acid–Alkali Digestion Method (Crude Fiber Method)(AOAC. 2000)
7.	**Fe content**	Atomic Absorption Spectrophotometry (AAS))(AOAC. 2000)
8.	**Zn content**	Atomic Absorption Spectrophotometry (AAS))(AOAC. 2000)
9.	**Pythic acid**	Phytic acid content in wheat flour, dough and chapatti will be determined according to the method of Latta and Eskin (1980) with slight modifications.
10.	**Phytase activity(Enzyme)**	Phytase activity in wheat flour will be determined following the method of Richardson *et al*. (2000) with slight modifications.
11.	**Bioavailability of Fe (%) and Zn (%)**	Simulated Digestion Method (James *et al.* 1981)
12.	**Nutritional ratio – Phytate Fe ratio and Phytate Zn ratio**	Atomic Absorption Spectrophotometry (AAS). (AOAC. 2000)
Experiment 2: Genotypic evaluation using SNP/ KASP markers associated with grain Fe and Zn content in wheat		
1	**Crop and genotype**	Wheat 18 genotypes will be selected from experiment no 1 (three high, three moderate, and three low content)
2	**Stage of sampling**	Seedling stage will be considered for experiment
3	**DNA Isolation**	CTAB Method (Doyle and Doyle, 1990)
4	**Design**	Appropriate molecular software will be used for SNP /KASP markers data analysis
5.	**SNP/ KASP Genotyping**	PCR- based amplification (for example Kompetitive allele-specific PCR: KASP) will be used for amplification of KASP markers. (Obermeier and Friedt 2015; Rasheed *et al*. 2017; You *et al*.2018;Schedben *et al* 2018Hickey *et al* 2017)

6.	**Targeted SNP/KASP Markers**	About 25 SNP/KASP Markers linked to Fe and Zn will be utilized for the genotypes of wheat genotype associated with grain Fe and Zn accumulation (Wang *et al* .2021)
7.	**Marker efficiency**	Comparison with phenotypes (Tuberosa, 2012).
8.	**Agarose gel electrophoresis**	Successful PCR amplified product of KASP /SNP markers will be separated using agarose gel electrophoresis. (Semagn *et al*. 2014)

Experiment 3: Expression profiling of candidate genes involved in Fe and Zn accumulation in wheat grains using qRT-PCR.		
1	**Crop and genotype**	18 selected wheat genotypes
2	**Stage of Sampling**	Grain developing stage (20-30 DDA after analysis)
4	**Design**	Appropriate molecular software will be used for gene expression analysis. .
5	**Replication**	3
6	**Observations to be recorded**	
RNA isolation and purification		Total RNA will be extracted from wheat grain samples using the TRIzol reagent method following the manufacturer's instruction (Chaparro-Encinas *et al*. 2020)
RNA quality check		agarose gel electrophoresis and spectrophotometric analysis.(Joseph *et al*. 2001)
cDNA synthesis		First-strand complementary DNA (cDNA) was synthesized from purified RNA using a reverse transcription kit according to the manufacturer's protocol (Abouseadaa *et al* 2025)

Selection of reference genes	GADPH/Actin specific gene will be utilized as reference (Wang *et al.*2019)
Fold change method	Up-regulation or down-regulation of selected genes in different wheat genotypes. Comparison of gene expression levels between high Fe/Zn genotypes and low Fe/Zn genotypes.
Selection of Candidate Genes	Candidate genes associated with ZIP (Wang *et al.*2019),NAS, YSL, Ferrition, NRAMP, (Borrill *et al* 2014;kumar et al,2019 ; Sharma *et al.*2019)
qReal-Time PCR	Gene expression analysis was performed using quantitative real-time PCR (qRT-PCR)(Ali-benali *et al* 2005)
Relative Gene Expression Analysis	Calculation of ΔCt (difference between target gene and reference gene). Calculation of ΔΔCt between control and treatment genotypes. Relative gene expression values calculated using the 2^-ΔΔCt method.

Statistical Analysis

The biochemical data obtained from the experiment with three replications will be statistically analyzed following the Complete Randomized Design (CRD). The analysis of variance will be performed using standard statistical procedures as described by (Panse and Sukhatme 1985)

The experimental data recorded for biofortified and normal wheat genotypes will be subjected to Analysis of Variance (ANOVA) to evaluate the significance of variation among the genotypes, as reported by (Thapa *et al*. 2022)

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